

## Cloning and Sequencing of a Plasmid-Borne Gene (*opd*) Encoding a Phosphotriesterase

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Plasmid pCMS1 was isolated from *Pseudomonas diminuta* MG, a strain which constitutively hydrolyzes a broad spectrum of organophosphorus compounds. The native plasmid was restricted with *Pst*I, and individual DNA fragments were subcloned into pBR322. A recombinant plasmid transformed into *Escherichia coli* possessed weak hydrolytic activity, and Southern blotting with the native plasmid DNA verified that the DNA sequence originated from pCMS1. When the cloned 1.3-kilobase fragment was placed behind the *lacZ'* promoter of M13mp10 and retransformed into *E. coli*, clear-plaque isolates with correctly sized inserts exhibited isopropyl- $\beta$ -D-thiogalactopyranoside-inducible whole-cell activity. Sequence determination of the M13 constructions identified an open reading frame of 975 bases preceded by a putative ribosome-binding site appropriately positioned upstream of the first ATG codon in the open reading frame. An intragenic fusion of the *opd* gene with the *lacZ* gene produced a hybrid polypeptide which was purified by  $\beta$ -galactosidase immunoaffinity chromatography and used to confirm the open reading frame of *opd*. The gene product, an organophosphorus phosphotriesterase, would have a molecular weight of 35,418 if the presumed start site is correct. Eighty to ninety percent of the enzymatic activity was associated with the pseudomonad membrane fractions. When dissociated by treatment with 0.1% Triton and 1 M NaCl, the enzymatic activity was associated with a molecular weight of approximately 65,000, suggesting that the active enzyme was dimeric.

Synthetic organophosphorus neurotoxins are used extensively as agricultural and domestic pesticides including insecticides, fungicides, and herbicides. Naturally occurring bacterial isolates capable of metabolizing this class of compounds have received considerable attention (20, 25) since they provide the possibility of both environmental and in situ detoxification (reviewed in reference 18). *Pseudomonas putida* MG and *Flavobacterium* spp. have been shown to possess the ability to degrade an extremely broad spectrum of organophosphorus phosphotriesters as well as thiol esters (4, 6). Recently, certain mammalian neurotoxins, such as diisopropyl phosphonofluoridate (1) and Soman (1,2,2-trimethylpropyl-methylphosphonofluoridate; J. DeFrank, personal communication), have been shown to be hydrolyzed by selected bacteria. Several of the bacterial strains possess constitutively expressed phosphotriesterases with broad substrate ranges including many commonly used organophosphorus pesticides (4, 6). None of these strains has shown the ability to utilize these neurotoxins as sole nutrient or energy sources, thus making mutant selection difficult (C. S. McDaniel and J. R. Wild, Arch. Environ. Contam. Toxicol., in press). The hydrolysis of organophosphorus compounds by the pseudomonad phosphotriesterase has been shown to proceed via nucleophilic addition of a molecule of water across the acid anhydride bond (V. E. Lewis, W. J. Donarski, J. R. Wild, and F. M. Raushel, Biochemistry, in press). (The class of enzymes EC 3.1.3 [which includes diisopropyl phosphorofluoridase and somanase] to which the *opd* gene product belongs was recently renamed "organophosphorus acid anhydride" at the 1987 DFPase Workshop held at Woods Hole Marine Biological Laboratories, Woods Hole, Mass. Synonyms which have been used include phosphotriesterase, parathion hydrolase, paraoxonase, and parathion aryl esterase.) In addition, applications

of enzymatic hydrolysis have been limited due to lack of economical fermentations of the native soil bacteria (19).

Two bacterial strains from the closely related genera *Pseudomonas* and *Flavobacterium* encode organophosphorus-degrading genes (*opd*) on large plasmids (40 to 65 kilobases [kb]) (15, 23, 24), while the locations of the degradative genes are unknown in other bacteria (13, 22). In the present study, the *opd* gene from *Pseudomonas diminuta* was sequenced and its membrane-associated gene product was expressed in heterologous genetic backgrounds from several promoter systems. The native enzyme has been partially purified, allowing molecular weight estimation, and the open reading frame has been verified by direct amino acid sequence of a purified  $\beta$ -galactosidase fusion polypeptide.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** *P. diminuta* MG is the original host of pCMS1 and was obtained from the laboratory of D. Gibson. *Escherichia coli* strains HB101-4442 (auxotrophic for uracil and proline; 10) and JM103 were used as host cells for the cloning vectors, pBR322 (3) and phage M13mp10 (14), respectively. The recombinant plasmid pBR322-038 contained a 1.3-kb *Pst*I fragment of pCMS1 cloned into the ampicillin resistance gene of pBR322. M13mp-038/008 and M13mp10-038/004 were oppositely oriented phage constructions which were enzymatically active or inactive, depending upon the orientation of the 1.3-kb fragment of pBR322-038. Hybrid gene fusions were produced in plasmid pMC1403 and expressed in *E. coli* CQ4 (28).

**Media and growth conditions.** Cultures were grown at 32°C (*P. diminuta*) or 37°C (*E. coli*). Nutrient medium consisted of 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), and 5 g of NaCl per liter (TYE). TF minimal medium (17) was used for *E. coli* strains and was supplemented with uracil (50  $\mu$ g/ml), proline (25  $\mu$ g/ml), vitamin B<sub>1</sub> (0.01%),

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Casamino Acids (0.1%), glucose (0.2%), and antibiotics (25 to 50 µg/ml) as required.

**Isolation of plasmid DNA.** Standard protocols for the isolation of DNA from *E. coli* for plasmid (7) or phage (14) have been previously described. Isolation of predominantly covalently closed circular plasmid DNA from *P. diminuta* was accomplished via a mild lysis procedure modified from that of Berns and Thomas (2).

**Cloning and sequencing of *opd* from the native plasmid.** The *Pst*I restriction fragments of pCMS1 were inserted into pBR322, inactivating the ampicillin gene (Focus 5:3, Bethesda Research Laboratories [BRL], Gaithersburg, Md., 1983). The resulting recombinant plasmids were used to transform competent HB101-4442, and tetracycline-resistant (*Tc*<sup>r</sup>) colonies were selected and evaluated for ampicillin sensitivity (*Ap*<sup>s</sup>). The plasmid structure of selected *Tc*<sup>r</sup> *Ap*<sup>s</sup> transformants was determined, and clones representing the different inserts were analyzed for activity.

The 1.3-kb *Pst*I insert of pBR322-038 was excised from its vector, purified by preparative agarose gel electrophoresis using a modified freeze-squeeze phenol procedure (S. A. Benson, *Biotechniques* March/April:66-67, 1984), and subsequently introduced into the multiple cloning site of M13mp10. The resulting recombinant molecules were transformed into competent *E. coli* JM103 cells, and clear-plaque isolates were selected. All subsequent manipulations of viral recombinant DNAs were performed according to the methodology of the BRL "M13 Cloning/Dideoxy Sequencing Manual." A variety of 5' and 3' deletions of pBR322-038 were constructed, using various restriction sites surrounding the *opd* gene (*Bam*HI, *Ava*I, *Nru*I, *Sal*I, *Sph*I). In addition, 3' exonuclease III deletions were utilized to identify gene boundaries.

Dideoxy sequencing was accomplished by the method of Sanger as detailed in the BRL "M13 Cloning/Dideoxy Sequencing Manual." In cases where GC compaction was evident, reverse transcriptase as well as the Klenow fragment of DNA polymerase was used (BRL, manufacturer's protocols). Oligonucleotide primers were synthesized using phosphoramidite chemistry with an Applied Biosystems Synthesizer according to the manufacturer's recommendations.

The 5' region of the *opd* gene was subcloned into the β-galactosidase gene for the purposes of producing a *lacZ* fusion polypeptide. The 1.3-kb *opd* fragment was restricted with *Ava*I (see Fig. 3); the staggered restriction fragment was end-filled with DNA polymerase (Klenow fragment) and blunt-end ligated into the 5' *Sma*I cloning site of the *lacZ* fragment of pMC1403 (28). This hybrid genetic construction was transformed into *E. coli* CQ4 (5).

**Production of *opd* probes and Southern DNA hybridization.** Various constructions containing the *opd* gene sequence (pCMS1, pBR322-038, M13mp10-038/008, and the inactive M13mp10-038/004) were evaluated for hybridization with the *opd*-containing fragment. Undigested controls and corresponding *Pst*I-digested samples were electrophoresed on a 0.7% agarose-TBE gel (89 mM Tris base, 89 mM borate, and 2.5 mM sodium EDTA). After photography, the gels were transferred (26) onto nitrocellulose paper and probed with <sup>32</sup>P-labeled nick-translated pBR322-038 DNA.

**Phosphotriesterase assay.** Routine analysis of parathion hydrolysis in whole cells was accomplished by suspending cultures in 10 mM Tris hydrochloride (pH 8.0) containing 1.0 mM sodium EDTA (TE buffer). Cell-free lysates were assayed using sonicated extracts as described previously (10) in 0.5 ml of TE buffer. The suspended cells or cell extracts

were incubated with 10 µl of substrate (100 µg of parathion in 10% methanol), and *p*-nitrophenol production was monitored at a wavelength of 400 nm. To induce the gene under *lac* control, 1.0 µmol of isopropyl-β-D-thiogalactopyranoside (Sigma) per ml was added to the culture media.

**Column chromatography, affinity chromatography, and protein sequencing.** *P. diminuta* cells from a 200-liter fermentation (grown in the National Institutes of Health-Department of Energy-sponsored fermentation facility of the Department of Biochemistry and Biophysics, Texas A&M University) were harvested by a continuous-flow centrifuge and suspended in 2.0 liters of 1.0 M NaCl. Samples of this suspension were agitated in a Waring blender for 30 s, and the resulting suspension was centrifuged at 400 × *g* for 10 min. Portions of this suspension (5.0 ml) were sonicated, treated with 0.1% Triton X-100, and stirred at room temperature for 2 h before chromatography.

The molecular weight of the native enzyme was determined by ascending Sephadex G-200 chromatography in the presence of 50 mM CHES buffer [2-(*N*-cyclohexyl-amino)ethanesulfonic acid (pH 9.0)] at 4°C. Enzymatic activity was located by introducing 50-µl aliquots of column fractions (2.0 ml) into a reaction volume of 0.8 ml containing 0.2 mM paraoxon and 50 mM CHES buffer (pH 9.0).

Purification of hybrid β-galactosidase proteins encoding the 5' region of the *opd* gene was achieved by immunoaffinity chromatography (28) and preparative gel electrophoresis. Gas-phase sequencing of the purified fusion polypeptide (Applied Biosystems 470A Sequencer, Applied Biosystems 120A On-line-PTH Analyzer, TAES Biotechnology Support Laboratory) was accomplished by the methods of Hewick et al. (12).

## RESULTS

**Partial purification and molecular weight estimation.** Upon cellular disruption of the native *P. diminuta* strain by sonication or French pressure cell disruption, 80 to 90% of the activity was associated with the particulate fraction. It was possible to release activity from the particulate complex by treatment with 0.1% Triton X-100 or 0.2% Tween 20 without significant loss of activity. When these enzyme preparations were analyzed by Sephadex G-200 column chromatography, the molecular weight of the enzymatically active fractions was 60,000 to 65,000.

**Cloning of pCMS1 into pBR322.** The entire DNA from the degradative plasmid was digested with *Pst*I (generating fragments of approximately 18.5, 17.3, 5.3, 4.3, 1.7, 1.6, 1.3, and 0.8 kb) and was subcloned into pBR322 within that vector's ampicillin gene. Cell-free lysates of *Ap*<sup>s</sup> clones selected from the *Tc*<sup>r</sup> transformants of *E. coli* HB101-4442 were tested for activity. One single-colony isolate was selected for its ability to hydrolyze parathion, and the expected phenotype (*Tc*<sup>r</sup> *Ap*<sup>s</sup>; auxotrophy for uracil and proline; parathion hydrolysis) was verified. A 5.6-kb, CsCl-purified plasmid was isolated from this strain and used to transform competent HB101-4442 cells, regenerating the phenotype and demonstrating that the hydrolytic activity was mediated by the recombinant plasmid. Other isolates with a similarly sized insert but lacking the hydrolytic activity were subsequently shown to have an orientation opposite to that of the active clone (data not shown). This observation demonstrated that the orientation of the *opd*-containing fragment within the pBR322 vector was critical to heterologous expression. Thus, it appeared that the expression of the 1.3-kb fragment (approximately 1 to 2% of the



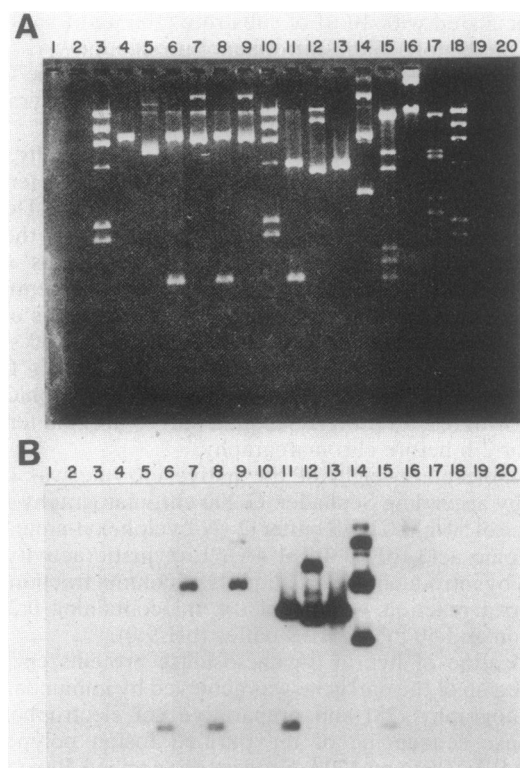


FIG. 1. Southern hybridization of *opd* probe (pBR322-038; pBR322 plus the 1.3-kb *opd* fragment) with the native plasmid and subclones. (A) Agarose gel electrophoresis of *opd*-containing DNAs. Lanes 6, 8, 11, and 15 contain *Pst*I-digested M13mp10-038/004 (inactive orientation), M13mp10-038/008 (active orientation), pBR322-038, and pCMS1, respectively. Lanes 7, 9, 12, and 16 contain the same unrestricted DNAs. The two cloning vectors used were included in lanes 4 and 5 (M13mp10) and in lanes 13 and 14 (pBR322) (restricted and unrestricted, respectively). Various restricted lambda DNAs were used as a molecular size marker in lanes 3, 10, 17 and 18. Two empty lanes occur on either side of the gel (lanes 1, 2, 19, and 20). (B) Southern blot of the gel in panel A to which <sup>32</sup>P-labeled *opd* (nick-translated pBR322-038) was hybridized. The lanes correspond to those described for panel A.

native *Pseudomonas* background) resulted from utilizing the ampicillin gene promoter of the vector.

Insertion of the 1.3-kb *Pst*I fragment into the multiple cloning site of M13mp10 produced an *opd*-encoding phage (M13mp10-038/008) possessing an inducible (isopropyl- $\beta$ -D-thiogalactopyranoside) whole-cell activity in *E. coli* JM103. Parathion was hydrolyzed by the phage-infected cells with a specific activity of approximately 10% of that of the native pseudomonad. This phage was used in hybridization studies ("C-tests") to select other isolates which possessed similarly sized insertions but lacked activity. In all cases, strains with hydrolytic activity gave negative C-tests with other active clones ("M13 Cloning/Dideoxy Sequencing Manual," BRL) (data not shown). Each of the negative isolates tested (M13mp10-038/003 and M13mp10-038/004) demonstrated positive C-test hybridization with the active clones, indicating that they contained the *opd* gene in the opposite orientation. These data were consistent with directional information provided by the pBR322 cloning.

**Southern blotting with *opd* probe.** Figure 1 summarizes the results of Southern hybridization of the *opd*-encoding replicons with <sup>32</sup>P-labeled, nick-translated pBR322-038 DNA

(26). In each case, the clones which exhibited hydrolytic activity (or which had been previously shown to possess that sequence in the opposite orientation) hybridized to the probe (lanes 6 through 9, 11 and 12, and 15 and 16). Lanes 6, 8, and 11 of Fig. 1 demonstrate that each clone containing *opd* regenerated a 1.3-kb fragment which comigrated with the same sized fragment of the native plasmid (lane 15). These studies verify that the native plasmid encoded a plasmid-mediated, parathion-degrading activity on a 1.3-kb *Pst*I fragment.

**Nucleotide sequencing.** Dideoxy sequencing along both strands of the *opd* gene revealed a potential translational reading frame of 975 base pairs, and the DNA sequence verified the known restriction pattern for the *opd*-encoding fragment (Fig. 2). Five oligonucleotide primers were constructed for the purposes of sequencing regions lacking convenient restriction sites. In all cases, these primers efficiently promoted DNA synthesis.

The open reading frame (CTC-GGC-ACC) began 12 base pairs from the 5' *Pst*I site and continued to a position at 1,038 base pairs before encountering a pair of closely spaced TGA stops (Fig. 3). A potential start site (ATG) was located 17 codons into the open reading frame. This codon appeared to be a candidate for the translational start since it is preceded by an AAGCAA sequence 15 base pairs upstream; the sequence and spacing are in good agreement with known *Pseudomonas* ribosomal binding sites (11, 15). In addition, several potential Rho-dependent terminator structures ranging in free energy of association from -12.6 to -15.4 kcal/mol (ca. -52.7 to -64.4 kJ/mol) were located 3' of the open reading frame (data not shown).

This predicted amino acid sequence would give rise to a protein of 35,418 daltons before posttranslational modifications, if any. However, there are other potential start sites further into the sequence which would give rise to slightly smaller proteins (Fig. 3). In particular, valine 7 represents a possible start since GTG (formylmethionyl) codons are known in *Pseudomonas* spp. (8) and since a potential ribosomal binding site was located for this start site. However, the insertion of a *Bam*HI linker into the *Sph*I site (Fig. 2) disrupted the first putative ATG translational start site, and these genetic constructions possessed no enzymatic activity.

**Amino acid sequencing of fusion polypeptides.** When a fusion protein was constructed between the 5' region of the *opd* gene and the *lacZ* gene at the *Ava*I-*Sma*I site, a hybrid polypeptide was recovered, purified, and subjected to amino acid sequencing. Amino acid sequencing confirmed the

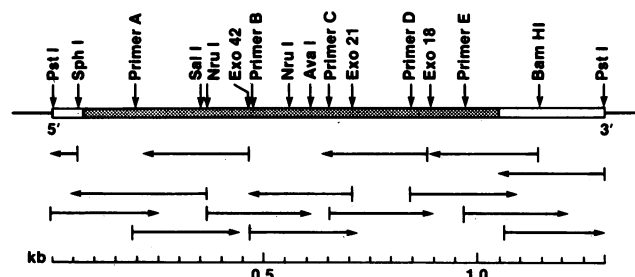


FIG. 2. Restriction map and DNA sequence strategy for the 1.3-kb fragment containing the *opd* gene. The direction and length of sequence determinations are shown with arrows (kilobase scale at bottom). The placement of the various restriction sites, exonuclease-generated subclones, and synthetic DNA primers used in the sequencing is shown along the fragment. The putative coding region for the *opd* gene is shaded.

Nucleotide Number	5'	CT	GCA	GCC	TGA	CTC	GGC	ACC	AGT	CGC	TGC	AAG	CAG	AGT	CGT	AAG	CAA	TCG	CAA	GGG	GGC	Amino Acid Number
60-119	AGC	Met	Gln	Thr	Arg	Arg	Val	Val	Glu	Lys		TCT	GCG	GCC	GCG	AGA	ACT	CTG	CTC	GGC	Gly	1-19
120-179	CTG	GCT	GCG	TGC	GCG	ACG	TGG	CTG	Leu	CGA		TCG	GCA	CAG	GCG	ATG	CGA	TCA	ATA	CGT	GCG	20-39
180-239	CGT	CCT	ATC	ACA	ATC	TCT	GAA	GCG	GGT	TTC		ACA	CTG	ACT	CAC	GAG	GAC	ATC	TCG	GCA	GCT	40-59
240-299	CGG	CAG	GAT	TCT	TGC	GTG	CTT	GGC	CAG	AGT		TCT	TCG	GTA	GCG	CAA	AGC	TCT	AGC	GGA	AAA	60-79
300-359	GGC	TGT	GAG	AGG	ATT	GCG	CGC	CAG	AGC	GGC		TGG	CGT	GCG	AAC	GAT	TGT	CGA	TGT	GTC	GAC	80-99
360-419	TTT	CGA	TAT	CGG	TCG	CGA	CGT	CAG	TTT	ATT		GGC	CGA	GGT	TTC	GCG	GGC	TGC	CGA	CGT	TCA	100-119
420-479	TAT	CTG	GCG	GCG	ACC	GCG	TTG	TGG	TTC	GAC		CCG	CCA	CTT	TCG	ATG	CGA	TTG	AGG	TAT	GTA	120-139
480-539	GAG	GAA	CTC	ACA	CTA	GTT	CTT	CCT	GCG	GTG		AGA	TTC	AAT	ATG	GCA	TCG	AAG	TAC	ACC	GGA	140-159
540-599	ATT	AGG	GCG	GGC	ATT	ATC	ATG	GTC	GCG	ACC		ACA	GGC	AAG	GCG	ACC	CCC	TTT	CAG	GAG	TTA	160-179
600-659	GTG	TTA	AAG	GCG	GCC	GCC	CGG	GCC	AGC	TTG		GCC	ACC	GGT	GTT	CCG	GTA	ACC	ACT	CAC	ACG	180-199
660-719	GCA	GCA	AGT	CAG	CGC	GAT	GGT	GAG	CGA	GGC		AGG	CCG	CCA	TTT	TTG	AGT	CCG	AAG	CTT	GAG	200-219
720-779	CCC	TCA	CGG	GTT	TGT	ATT	GGT	CAC	AGC	GAT		GAT	ACT	GAC	GAT	TTG	AGC	TAT	CTC	ACC	GCC	220-239
780-839	CTG	CTG	CGC	GGA	TAC	CTC	ATC	GGT	CTA	GAC		CAC	ATC	CCG	CAC	AGT	GCG	ATT	GGT	CTA	GAA	240-259
840-899	GAT	AAT	GCG	AGT	GCA	TCA	CCG	CTC	CTG	GGC		ATC	CGT	TCG	TGG	CAA	ACA	CGG	GCT	CTC	TTG	260-279
900-959	ATC	AAG	GCG	CTC	ATC	GAC	CAA	GGC	TAC	ATG		AAA	CAA	ATC	CTC	GTT	TCG	AAT	GAC	TGG	CTG	280-299
960-1019	PTC	GGG	TTT	TCG	AGC	TAT	GTG	ACC	AAC	ATC		ATG	GAC	GTG	ATG	GAT	CGC	GTG	AAC	CCC	GAC	300-319
1020-1079	GGG	ATG	GCC	TTC	ATT	CAC	TGA	GAG	TGA	TCC		CAT	TCT	ACG	AGA	GAA	GGG	CGT	CCC	ACA	GGA	320-325
1080-1139	AAC	GCT	GGC	AGG	CAT	CAC	TGT	GAC	TAA	CCC		GGC	GCG	GTT	CTG	TGT	CAC	CGA	CTT	GCC	GTG	
1140-1199	CAT	GAC	GCC	ATC	TGG	ATC	CTT	CAC	CGC	AGC		GGC	CAC	TAT	TCC	CCG	TCA	AGA	TAC	CGA	ACG	
1200-1259	ATG	AAG	TCG	CGC	ATC	GAT	AGG	CAT	CTT	CAA		TGT	GAT	CAG	GGC	TGC	CAC	CTC	CAA	AGC	CGG	
1260-1322	TGG	CAG	CCC	CTG	TCG	ATA	GTC	TTG	AGG	GAC		GGT	AGC	GAC	GAC	CGT	GCT	TTT	CGT	GAA	CTG	

FIG. 3. Nucleotide sequence of the *opd* gene fragment. The amino acid sequence corresponding to the open reading frame beginning with the first ATG codon is identified below the sequence. Primers used in the sequencing are shown above the nucleotide sequence by overlining. The 3' stop codon is indicated with a period. The amino acids confirmed by protein sequencing are underlined.

predicted reading frame for 16 amino acids 5' of the fusion junction (Fig. 3). The sequence is 168 amino acids away from the presumed translational start for the *opd* gene product; however, truncated polypeptides are typical of fusions of membrane proteins with  $\beta$ -galactosidase (28), and proteolysis in the heterologous background may have produced a posttranslationally modified polypeptide.

**Subcloning regional deletions.** Figure 4 summarizes results obtained with various subclones of the 1.3-kb fragment containing the *opd* gene. Deletions outside the putative coding region remained active when the sequence was properly oriented for expression from the *lacZ* promoter. If the orientation was reversed or if deletions were made within the putative coding region, activity was eliminated.

## DISCUSSION

The gene (*opd*) encoding a broad-substrate-range phosphotriesterase of *P. diminuta* MG has been shown to be encoded on a 50- to 60-kb plasmid (15, 23, 24; C. S. McDaniel, Ph.D. dissertation, Texas A&M University, College Station, 1985). The plasmid-borne gene was contained within a 1.3-kb restriction fragment and was transferred into a variety of plasmid and phage vectors and expressed in *E. coli*. The 1.3-kb fragment encoding the *opd* gene was sequenced, and its proper reading frame was confirmed by

protein sequencing. The *opd* gene contained within the 1.3-kb fragment of the native plasmid possessed an open reading frame of 325 codons preceded by a 5' flanking region with translational signals typical of other bacterial genes (8, 11, 16). In addition, the disruption of the presumed translational start site by the insertion of a *Bam*HI linker destroyed phosphotriesterase production. The predicted size of the deduced gene product and the size limitations defined by subcloned fragments are consistent with a predicted monomeric molecular weight of 35,418. Column chromatography of a detergent-treated cell extract demonstrated an enzymatic activity at 60,000 to 65,000  $M_r$  which suggests that individual monomers might dimerize to form a holoenzyme. A similar phosphotriesterase has been described from *Flavobacterium* sp. strain ATCC 27551 (4), in which the hydrolytic activity was associated with a protein estimated to be greater than 50,000  $M_r$ .

Restricted applications of chlorinated hydrocarbon pesticides, as the result of their inherent environmental hazards, have led to increased use of carbamate and organophosphorus pesticides. However, these applications have been compromised by the presence of soil bacteria capable of rapidly degrading the organophosphorus compounds (21). The potential transfer of plasmid-mediated pesticide detoxification genes through a variety of hosts has important

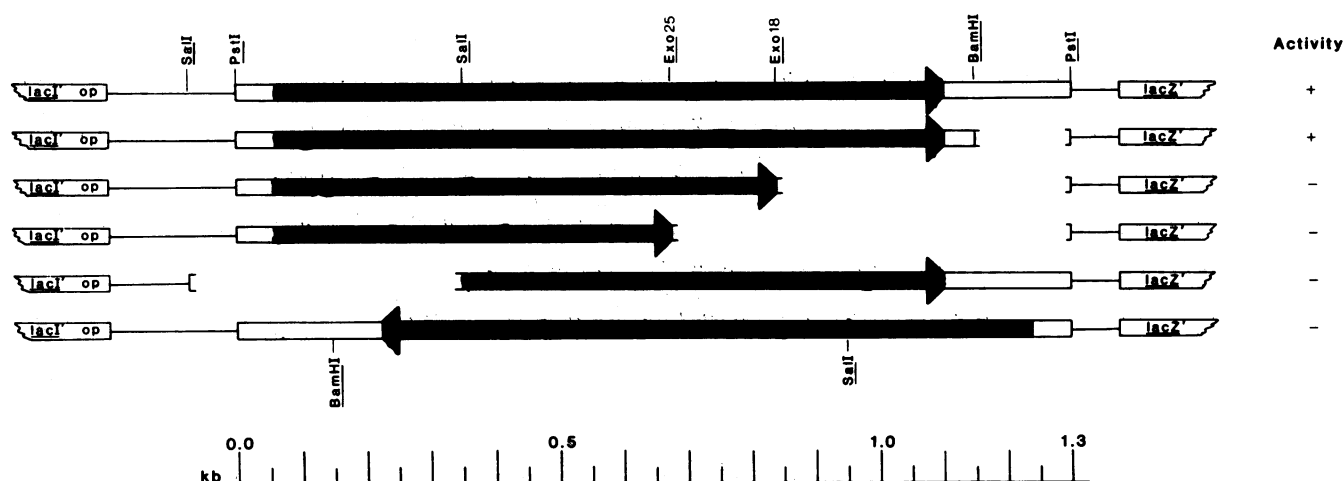


FIG. 4. Activity of *opd* subclones. M13 constructions in which the *opd* gene was placed under control of the *lac* promoter are shown. Sequences are adjusted to align vector DNA on either side of the *opd* subclone. Deletions are indicated by open space between brackets. The putative coding region for the *opd* gene is indicated by shading, and the sense direction is shown by arrows.

implications relative to the loss of efficacy of these biolabile pesticides. The potential mobility of these plasmid-borne genes may be analogous to the reduction of antibiotic efficacy in clinical and agricultural situations by plasmid-borne resistance factors (29).

It is clear that many soil bacteria possess degradative, plasmid-borne genes which could be readily transferred and expressed among a variety of bacterial and viral hosts. This phenomenon is not limited to organophosphorus neurotoxins, since plasmid-borne genes for degradative enzymes of herbicides have been well documented (9, 27). In the case of the *opd* genes, a wide range of pesticides sharing a common chemical structure are degraded (6), providing the potential for rapid evolution of genes to degrade a variety of pesticides and challenging the agrochemical rationale of substituting pesticides of similar chemical structure or increasing application rates for extended pest control. Rapid mutational adaptation in an enriched soil bacterial population could render ineffective any subsequent applications of a similar chemical.

#### LITERATURE CITED

- Attaway, H., J. O. Nelson, A. M. Baya, M. J. Voll, W. E. White, D. J. Grimes, and R. R. Colwell. 1987. Bacterial detoxification of diisopropyl fluorophosphate. *Appl. Environ. Microbiol.* 53: 1685-1689.
- Berns, K. I., and C. A. Thomas. 1965. Isolation of high molecular weight DNA from *Hemophilus influenzae*. *J. Mol. Biol.* 11:476-490.
- Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Croso, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2:95-113.
- Brown, K. A. 1980. Phosphotriesterases of *Flavobacterium* sp. *Soil Biol. Biochem.* 12:105-112.
- Casadaban, M. J., J. Chou, and S. N. Cohen. 1980. In vitro gene fusions that join an enzymatically active  $\beta$ -galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. *J. Bacteriol.* 143:971-980.
- Chiang, T., M. C. Dean, and C. S. McDaniel. 1985. A fruitfly bioassay for detection of certain organophosphorous insecticide residues. *Bull. Environ. Contam. Toxicol.* 34:809-814.
- Clewell, D. B., and D. R. Helinski. 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. *Proc. Natl. Acad. Sci. USA* 62:1159-1166.
- Diver, W. P., J. Grinstead, D. C. Fritzinger, N. L. Brown, J. Altenbuchner, P. Rogowsky, and R. Schmitt. 1983. DNA sequences of and complementation by the *tnpR* genes of Tn21, Tn501, Tn1721. *Mol. Gen. Genet.* 191:189-193.
- Fisher, P. R., J. Appleton, and J. M. Pemberton. 1978. Isolation and characterization of the pesticide-degrading plasmid pJP1 from *Alcaligenes paradoxus*. *J. Bacteriol.* 135:798-804.
- Foltermann, K. F., M. S. Shanley, and J. R. Wild. 1984. Assembly of the aspartate transcarbamoylase holoenzyme from transcriptionally independent catalytic and regulatory cistrons. *J. Bacteriol.* 157:891-898.
- Frantz, B., and A. M. Chakrabarty. 1987. Organization and nucleotide sequence determination of a gene cluster involved in 3-chlorocatechol degradation. *Proc. Natl. Acad. Sci. USA* 84: 4460-4464.
- Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer. 1981. A gas-liquid, solid phase peptide and protein sequencer. *J. Biol. Chem.* 256:7990-7996.
- Meritt, G. C., J. E. Watts, and K. McDougall. 1981. In vitro degradation of organophosphorous insecticides by *Pseudomonas aeruginosa* isolated from fleece rot lesions of sheep. *Austral. Vet. J.* 57:531.
- Messing, J., B. Gronenborn, B. Muller-Hill, and P. H. Hofschneider. 1977. Filamentous coliphage M13 as a cloning vehicle: insertion of a *Hind* III fragment of the *lac* regulatory region in M13 replicative form in vitro. *Proc. Natl. Acad. Sci. USA* 74: 3642-3646.
- Mulbry, W. W., J. S. Karns, P. C. Kearney, J. O. Nelson, C. S. McDaniel, and J. R. Wild. 1986. Identification of a plasmid-borne parathion hydrolase gene from *Flavobacterium* sp. by Southern hybridization with *opd* from *Pseudomonas diminuta*. *Appl. Environ. Microbiol.* 51:926-930.
- Mullin, D., S. Minnich, L. S. Chen, and A. Newton. 1987. A set of positively regulated flagellar gene promoters in *Caulobacter crescentus* with sequence homology to the *nif* gene promoters of *Klebsiella pneumoniae*. *J. Mol. Biol.* 195:939-943.
- Munch-Petersen, A., and J. Neuhaard. 1964. Studies on the acid-soluble nucleotide pool in thymine-requiring mutants of *Escherichia coli* during thymine starvation. I. Accumulation of deoxyadenosine triphosphate in *Escherichia coli* 15TAU<sup>-</sup>. *Biochim. Biophys. Acta* 80:542-551.
- Munnecke, D. M. 1981. The use of microbial enzymes for pesticide detoxification, p. 251-270. In T. Leisinger, A. M. Cook, R. Hutter, and J. Nuesch (ed.), *Microbial degradation of xenobiotics and recalcitrant compounds*. Academic Press, Inc. (London), Ltd., London.
- Munnecke, D. M., and H. F. Fischer. 1979. Production of



- parathion hydrolase activity. Eur. J. Appl. Microbiol. **8**:103–112.
20. Munnecke, D. M., and D. P. H. Hsieh. 1974. Microbial decontamination of parathion and p-nitrophenol in aqueous media. Appl. Microbiol. **28**:212–217.
21. Read, D. C. 1983. Enhanced microbial degradation of carbofuran and fensulfothion after repeated applications to acid mineral soil. Agric. Ecosystems Environ. **10**:37–46.
22. Rosenberg, A., and M. Alexander. 1979. Microbial cleavage of various organophosphorous insecticides. Appl. Environ. Microbiol. **37**:886–891.
23. Serdar, C. M., and D. T. Gibson. 1985. Enzymatic hydrolysis of organophosphates: cloning and expression of a parathion hydrolase gene from *Pseudomonas diminuta*. Bio/Technology **3**:567–571.
24. Serdar, C. M., D. T. Gibson, D. M. Munnecke, and J. H. Lancaster. 1982. Plasmid involvement in parathion hydrolysis by *Pseudomonas diminuta*. Appl. Environ. Microbiol. **44**:246–249.
25. Sethunathan, N., and T. Yoshida. 1973. A *Flavobacterium* sp. that degrades diazinon and parathion. Can. J. Microbiol. **19**:873–875.
26. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
27. Stalker, D. M., and K. E. McBride. 1987. Cloning and expression in *Escherichia coli* of a *Klebsiella ozaenae* plasmid-borne gene encoding a nitrilase specific for the herbicide bromoxynil. J. Bacteriol. **169**:955–960.
28. Struck, D. K., D. Maratea, and R. Young. 1985. Purification of hybrid  $\beta$ -galactosidase proteins encoded by  $\Phi$ X174 *E* $\Phi$ lacZ and *Escherichia coli* *prlA* $\Phi$ lacZ: a general method for the isolation of lacZ fusion polypeptides produced in low amounts. J. Mol. Appl. Genet. **3**:18–25.
29. Waid, J. S. 1973. The possible importance of transfer factors in bacterial degradation of herbicides in natural ecosystems. Residue Rev. **44**:65–71.